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Assessing the Qualitative Validity of a Non-Invasive Ultrasound Technique for Estimating Muscle Glycogen Change

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Assessing the Qualitative Validity of a Non-Invasive Ultrasound Technique for Estimating Muscle Glycogen Change

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Abstract

Assessing the Qualitative Validity of a Non-Invasive Ultrasound Technique for Estimating Muscle Glycogen Change

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Intramuscular glycogen is a primary fuel source for intense exercise of any substantial duration, and the use of an invasive muscle biopsy is still the gold-standard of measurement. The purpose of this study is to assess the qualitative validity of a proprietary algorithm (MuscleSound®) for determination of intramuscular glycogen concentration against the body of literature on muscle glycogen depletion from exercise and subsequent recovery. To test whether this new technology appears valid, 12 healthy recreational students (n=1 female) (25.5 ± 1.25y, 74.03 ± 2.58kg, 174 ± 4.48cm) were recruited from the University of Texas at Austin campus for participation in this study. Subjects performed six bouts of six-minute efforts on a stationary cycle ergometer at 84% of their VO_{2peak}; an exercise protocol designed to elicit substantial muscle glycogen depletion. Care was taken to maintain body hydration status to further elucidate whether the device was truly measuring glycogen or a proxy such as intramuscular water. Each interval was followed by a six-minute rest period, during which time ultrasound images were taken of the vastus lateralis (VL), rectus femoris (RF) and gastrocnemius/soleus (GS) muscles. Images were processed using the MuscleSound® (MS) algorithm which

assigns a MS score (0-100) based on the opacity of the image. Following the exercise protocol, subjects remained in the laboratory for a six-hour recovery phase, wherein images were taken at the same three sites at two-hour intervals. A high carbohydrate (CHO) and protein (PRO) beverage was consumed immediately following exercise, and after two and four hours in order to assess the MS algorithm's ability to capture the qualitative pattern of glycogen resynthesis established in the literature. It was hypothesized that the VL and RF muscles would decrease in a curvilinear fashion during the intervals to a greater extent than the GS and that during the recovery phase these muscles would show an increase in MS units every two hours for six hours. In support of our hypothesis, we found that during exercise, MS values decreased in a curvilinear pattern in the VL with significantly lower values after bouts 3 through 6 versus baseline. The RF showed a similar pattern with bouts 4 through 6 being significantly lower than baseline, while the GS remained constant throughout. After 120 minutes of recovery the MS values of the VL and RF had returned to baseline levels. It is established in the literature that a nearly depleted muscle should take approximately 24 hours to return to capacity (36); therefore it is unclear whether the MS technology is truly capturing muscle glycogen in its images. While an effective qualitative tool, more work should be done to determine if MS is a valid quantitative measurement of muscle glycogen.

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INTRODUCTION

The body relies almost entirely on the oxidation of fat and carbohydrates (CHO) for its metabolic needs during exercise lasting more than several minutes. CHOs, however, are the primary energy source for exercise at high intensities (17, 51) as the rate of fat oxidation is too low for fat to be the primary substrate during high intensity exercise (17). Fatty acid (FA) transport into the mitochondria is thought to be the rate limiting step in this process (16). Therefore, once corporeal reserves of glycogen and blood glucose are depleted physical activity is limited to that which can be sustained with the slower rate of fat oxidation (30-40% VO_2 max) (17). These muscle glycogen stores are dependent on dietary patterns and activity level, but are generally within the range of about 10-30g/kg (60-180 mmoles /kg) glycogen (16). Or, presented per 100 grams wet tissue, others have reported values between 1.54-2.16g/ 100g wet tissue (15). The relative percentage of CHO oxidation from muscle glycogen versus plasma glucose increases as exercise intensity increases (57). CHO's pivotal role as an energy source is demonstrated in a wide body of literature modeling the pattern of depletion in humans performing physical activity (4, 10-12, 15, 17, 20, 23, 33, 42, 50, 52).

Previous literature on muscle glycogen depletion has focused primarily on endurance exercise (9, 10, 12, 17, 19) as it is widely established that a primary determinant of the body's ability to continue prolonged strenuous exercise is the availability of muscle glycogen. In studies using cycling as the exercise modality, glycogen content in the vastus lateralis muscle (VL) is frequently sampled through the muscle biopsy technique (3). In one such study by Coyle *et al*. muscle glycogen concentration in the VL decreased from 163 ± 13 (mmol gu/kg) to 37 ± 5 (mmol gu/kg) or by 77% during 3 hours of cycling at 71.4 ± 0.8% VO_2 peak (17). Runners, on the other

hand, show smaller reductions in the VL compared to the gastrocnemius and soleus while running on a flat surface, but increased utilization while running uphill (12, 13). This may be due to the utilization of a smaller muscle mass when cycling when compared to running, and therefore greater metabolic stress placed on the VL (15). Thus, cycling was chosen as the exercise modality for the present study as previous work suggests it will provide the greatest decline in muscle glycogen.

Intermittent high intensity exercise has also been used as a tool for investigating muscle glycogen depletion patterns (23, 26). Intervals present a fitting modality to test for glycogen depletion as incorporating a rest period allows for the subsequent exercise to be performed at a higher work rate. As the work rate increases there is a commensurate increase in muscle fiber activation as predicted by the size principle of motor neuron recruitment (32). This facilitates depletion of glycogen in the fast twitch glycolytic fibers that otherwise might not have been activated at lower intensities.

Studies suggest that the pre exercise intramuscular glycogen content dictates the extent to which it is preferentially used in exercise (27, 42, 52, 62). An increase in glycogen reserves serves to increase the amount of CHO oxidized and glycogen depletion. To maximize this, classic glycogen loading regimens call for three days of high intensity exercise with low dietary CHO followed by two days of low intensity exercise and a high CHO diet (52).

Post exercise replenishment of muscle glycogen generally falls into the range of about 5% per hour or 5mmol/kg muscle/hr (16). The rate of replenishment plateaus when about 0.7-1.5g CHO/kg body mass are consumed per 2hr (36, 37, 63). High glycemic index sources of CHO such as glucose and sucrose are more effective than fructose for replenishment (16). If ingestion of CHOs begins promptly after the cessation of exercise then the rate of glycogen resynthesis can be increased to 7-8mmol/kg/hr for the first 2

hours (16, 36). However, a study by Ivy *et al*. has shown that this rate can be increased to 15 mmol/l/hr for two hours when protein is included in the CHO beverage (40).

The purpose of the present study is to investigate the qualitative validity of a proprietary algorithm (MuscleSound®) for non-invasive determination of muscle glycogen concentration using high-frequency ultrasound imaging by comparing the results to the expected patterns in the literature. We hypothesize that if the algorithm is measuring muscle glycogen or a proxy of muscle glycogen, the VL and RF MS units should decrease in a curvilinear pattern due to the interval bouts, and thereafter show an increase every two hours for six hours post exercise during the replenishment trial (40).

REVIEW OF LITERATURE

CHO Storage and Metabolism

The amount of muscle glycogen stored in the body is dependent on diet and exercise. Glycogen storage ranges from 10-30g/kg (60-180mmol/kg) skeletal muscle, with an additional 80g of glycogen stored in the liver, thus providing for approximately 8400KJ of energy (16). Within the muscle, glycogen exists in two forms: as the acid soluble macroglycogen, or the acid insoluble proglycogen. Proglycogen has been shown to display greater rates of resynthesis following CHO supplementation (1). Glycogen in the liver is broken down in the process of glycogenolysis and transported as plasma glucose to be used in active muscles (16). As the supply of endogenous CHO in the form of muscle and liver glycogen and plasma glucose is limited, depletion of these sources leads to fatigue (9, 17), emphasizing its critical role as a substrate for maintaining high work rates.

During moderate-intensity exercise (i.e. 60-75% VO_2max) fat provides only about half of the energy with carbohydrates providing the balance (16). At high intensities of exercise (i.e. 85% VO_2max), muscle glycogen breakdown and plasma glucose provide greater than two-thirds of the energy required (16, 50, 51). These observations were in subjects who had fasted for 12hr prior to testing, as it is known that CHO ingestion prior to exercise increases the percentage of energy derived from this substrate (16, 18, 61, 62).

Previous work by Coyle *et al*. has demonstrated that during prolonged moderate intensity exercise (60-80% VO_2 max) muscle glycogen is depleted, and fatigue occurs as a result (17, 19). In this study fatigue was delayed by one hour with exogenous CHO

ingestion, enabling the body to utilize predominantly plasma glucose to meet its energy needs (17, 19). During the early portion of exercise, muscle glycogen is the predominant substrate, while plasma glucose's contribution to metabolic energy expenditure increases with exercise duration (50). Additionally, as exercise intensity increases tissue uptake of plasma glucose and muscle glycogen oxidation increase (50).

FAT STORAGE AND METABOLISM

Fat stores are the largest reserve of energy within the body, totaling 200-627 MJ (16). Triglycerides are stored as intramuscular triglycerides (IMTG) or within adipose tissue dispersed throughout the body. Triglyceride stores must be hydrolyzed in the process of lipolysis before they can be utilized for energy. Hydrolysis of adipose tissue allows free fatty acids (FFA) to subsequently be transported in the plasma bound to albumin. Within muscle, IMTGs are hydrolyzed into FA to be oxidized in the mitochondria.

The body's oxidation of fat varies based on the intensity of exercise, but in general lower intensity exercise utilizes a much greater percentage of fat for energy requirements than higher intensity exercise (50). At 70% VO_2 max the body can only oxidize fat to meet half of its energy requirement, and at 85% VO_2 max this decreases to a third (16). When CHO stores are depleted people must resort to exercise that can be met by only fat oxidation (i.e. 30-50% VO_2 max) (16, 17, 19). This limitation is not due to a lack of plasma FFA availability as studies show that at the point of fatigue, when CHO are depleted and the body is relying primarily on fat oxidation, there are sufficiently high levels of plasma FFA (17, 19). Thus, the muscle's ability to oxidize fat is thought to

be the rate limiting step, since raising the plasma FFA concentration does not increase energy expenditure (31).

One theory behind the body's limited ability to oxidize fat has to do with CHO availability. Increased conversion of CHO to fat leads to the intermediate malonyl-CoA, which has been shown to inhibit carnitine palmitoyltransferase (CPT), the transporter protein responsible for transport of FFA into the mitochondria for subsequent oxidation (16). Additionally, at lower intensities lipolysis of fat from adipose tissue is stimulated maximally, but as exercise intensity increases blood flow is shunted away from adipose tissue (50). Since less blood flow reaches the adipose tissue, there is a diminished ability for albumin to carry FFA to the working muscle cells with a concurrent reduction of plasma fatty acid oxidation (50). IMTG lipolysis and oxidation still represents a small fraction of overall substrate oxidation as muscle glycogen is the predominant substrate at high intensities (50). Over time, however, there is an increased utilization of plasma-derived substrate oxidation as intramuscular fuels are diminished (50). At the cessation of exercise there is a decrease in overall lipolysis but an increase in plasma fatty acid content as blood flow is restored to the peripheral adipose tissue and albumin is made available for transport (50). This increased rate of release of fatty acids into the plasma is a direct result of already hydrolyzed triglycerides (50).

GLYCOGEN UTILIZATION DURING ENDURANCE EXERCISE

Muscle glycogen depletion in physical activity has been modeled extensively in the literature due to glycogen's importance as a metabolic substrate (2, 4, 10, 12, 17, 19, 30, 33, 42). As previously noted, glycogen is the primary fuel source for exercise at

moderate intensities or higher (16, 30, 50). In a classic study on muscle glycogen utilization, Hermansen *et al*. (1967) had trained and untrained subjects exercise to volitional exhaustion on a cycle ergometer while measuring their respiratory quotient (RQ) (33). They measured glycogen in the VL before and after the bout of cycling and found that the muscle glycogen content decreased from 1.6g/100g wet muscle to 0.06 g/100g wet muscle (33). Across the whole trial, they also found combustion of CHO to be on average 2.8 g/min, as determined from a RQ of 0.9 or higher (33). Glycogen depletion correlated with CHO oxidized, thus highlighting that muscle glycogen oxidation was limiting at high-sustained workloads (33). And further work by Gollnick *et al*. (1973) demonstrated that cycling at even greater workloads lead to amplified glycogen depletion, with Type I fibers being preferentially depleted at submaximal workloads (30). This finding has been corroborated and is widely supported in the literature (16, 50, 51).

Muscle activation differs between cycling and running, and work by Costill *et al*. helped to elucidate the muscle groups utilized during running (12). Muscle glycogen depletion patterns were assessed over the course of a 30km running race at 83% VO_2 max (12). Muscle glycogen declined 56% in the VL, and histochemical staining revealed that slow twitch fibers were preferentially depleted with only a small amount of depletion occurring in the fast twitch fibers (11).

Muscle glycogen depletion patterns change based on the number of days in a row of intense exercise as demonstrated by Costill and coworkers (10). They assessed muscle glycogen utilization over 3 consecutive days of a 16.1km treadmill run at 80% VO_2max

(10). Muscle glycogen utilization was greatest on the first run and decreased thereafter as determined by muscle biopsies (10). Measurement of lactate accumulation, glucose and FFA further revealed that each successive run was achieved with diminished CHO oxidation and increased reliance on lipid stores (10). The implications of this work apply to any athlete seeking to exercise intensely or race over several days. Since fat oxidation is rate limiting, unless the intramuscular stores of glycogen are replenished through high carbohydrate meals the athlete will be limited in his ability to race at high intensities.

Coyle *et al*. demonstrated the importance of CHO stores for maintaining high intensity endurance exercise in a study designed to examine whether CHO ingestion during prolonged cycling at 71.4% VO_2 max to exhaustion could delay the time to fatigue (19). In the study they found that in the group that was given CHO, blood glucose was 20-40% higher (19). CHO ingestion prevented the exercise induced drop in plasma insulin and allowed subjects to maintain RER. Fatigue was postponed in 7 of 10 subjects and the total amount of work performed was 6.6% higher in the CHO group (19).

Coyle *et al*. (1986) sought to elucidate the mechanism behind the findings from the previous (1983) study (17). To do this were either fed placebo or CHO during exercise at 71% VO_2 max to determine whether the slowing of fatigue when fed CHO was due to an attenuation of the decline in muscle glycogen. Profoundly, they found that muscle glycogen utilization was nearly identical when fed CHO or placebo and that the last hour of exercise achieved in the CHO trial was due to high rates of sustained blood glucose oxidation (17). Despite high levels of blood glucose subjects inevitably fatigued and Coyle *et al*. concluded that some other mechanism caused the fatigue. Because the

pattern of muscle glycogen utilization was similar between treatment groups it is clear that muscle glycogen is preferentially utilized as an energy source early in exercise regardless of exogenous carbohydrate intake (17). In summary, the subjects that received CHO exercised one hour longer than those that received placebo, but this extra hour was achieved with little further depletion of muscle glycogen, as these sources were preferentially depleted early in exercise (17).

The findings from Coyle *et al*. (1986) highlighting the ability of CHO ingestion to delay fatigue were further explored to determine if indeed blood glucose is the main source of substrate for CHO oxidation late in exercise (9). Subjects cycled to a fatigued state in the first bout of exercise, then either received a placebo, a CHO beverage or an intravenous infusion of CHO to maintain euglycemia. Those subjects that were infused maintained a high rate of CHO oxidation and RER and subsequently cycled for an additional 45 min in bout two, whereas those that received the oral CHO cycled for an intermediate time than infusion and placebo (9). The high rate of CHO oxidation as displayed by RER was still lower than the first bout, suggesting that after sustained exercise there is a gradual shift to less ability to oxidize CHO, possibly indicating a role for the central nervous system in CHO oxidation late in exercise (9).

MUSCLE GLYCOGEN DEPLETION DURING HIGH INTENSITY INTERVAL EXERCISE

While muscle glycogen depletion is most commonly associated with endurance exercise lasting 2-3 hours, it is also the case that high intensity intervals of approximately 90-150% VO_2 max lasting only 15-30 total minutes can cause significant depletion (16, 44). Importantly, high-intensity intermittent exercise is a valid way to drastically decrease

muscle glycogen concentration as the resistance utilized necessitates the recruitment of fast-twitch Type II glycolytic fibers. Endurance protocols, conversely, cause an initial decrease in Type I oxidative fibers and only incorporate Type II fibers when the former become depleted.

Drastic declines in muscle glycogen in the VL have been shown after high intensity intermittent exercise of varying length, repetitions and intensities. Gollnick *et al*. 1973 used a protocol of six times one-minute efforts at a wattage corresponding to 150% of the subjects VO_2 max with 10 minutes rest in between repetitions (29). Muscle glycogen concentration declined as a function of the number of repetitions completed with Type II glycolytic fibers depleting predominantly. After the first bout alone, muscle glycogen in the VL had declined 20%. After the sixth and final work bout muscle glycogen had declined by 62.8% (29).

High-intensity interval studies of longer duration are just as effective for depletion of muscle glycogen as demonstrated in a study by Vollestad *et al*. (1985). Subjects in this study cycled for 10 minutes at 90% of VO_2 max with 5 minutes of rest in between efforts (60). They observed the largest decrease in glycogen in the VL was during the first 10 minutes of cycling (84, 75, 71, & 80% of post warm-up values; Type I, IIA, IIAB and IIB respectively) (60). And at exhaustion the VL values had decreased to 26, 20, 39 and 48% of post warm-up values respectively (Type I, IIA, IIAB and IIB) (60). Subjects in this study only completed three intervals, less total work time than in the current study.

Essen *et al*. (1978) directly compared glycogen depletion patterns in subjects performing intermittent versus continuous exercise over an hour. Subjects in the

intermittent trial performed 15 seconds of work with equal rest for 60 min at an intensity eliciting 100% of VO_2 max. In the continuous trial subjects cycled at 50% of the resistance used in the intermittent trial. Intermittent exercise resulted in a mean decrease of 213 and 203 mmol/kg dry weight (Type I and II fibers respectively). Whereas during the continuous exercise there was a mean decrease of 277 and 113 mmol/kg dry weight (Type I and II respectively) (23). Due to the greater utilization of Type II fibers in high intensity intermittent exercise, the present study used intermittent cycling to provide the most robust signal of muscle glycogen decline.

There is an abundance of literature on the effects of endurance exercise on glycogen, these studies highlight that intermittent high-intensity exercise is equally as effective at causing decreases. The higher workload in interval type training allows for an even greater rate of glycogen depletion than endurance exercise. Thus, for the purpose of ensuring a rapid rate of decline in muscle glycogen, intervals of 84% $VO_{2\ peak}$ were used in the present study.

MUSCLE WATER CONTENT

Approximately 3 grams of water are stored along with each gram of glycogen in skeletal muscle (48, 53). This was determined by Olsson and Saltin (1970) after they had subjects perform a heavy arm and leg resistance protocol while on a high fat and protein diet for three days, followed by a high CHO diet for 4 days (48). Measurements of muscle glycogen were done on the 3rd and 7th day of the CHO feeding trial. Muscle glycogen increased from 4.5 to 19.9 g/kg wet muscle from the 3rd to 7th day and total body water increased 2.2 liters. The increase in total body water was assumed to be due

to storage of glycogen in the muscle and liver. From these results they calculated that there were 3-4 grams of water per gram of glycogen, and that the deposition of water most likely is in the cells themselves. Further support for this phenomenon has been demonstrated in studies where as a result of three sets of three minutes of intense cycling at 120% VO_2 max total water content in the VL increased from 313 to 359ml/100g dry weight, and extracellular water increased from 34 to 60 ml/100g dry weight (54). In muscles not involved in the workload there was no change in water content (54). These studies provide a possible mechanism whereby the MuscleSound® technology evaluated in the current investigation may utilize intramuscular water content as a proxy for the glycogen molecules.

GLYCOGEN LOADING AND EFFECT ON UTILIZATION

The amount of glycogen stored in the muscle prior to exercise is a primary factor determining the ability to sustain high workloads and rates of carbohydrate oxidation (27). Greater reserves of muscle glycogen increases CHO oxidation, glycogen depletion, and lactate buildup. In their classic study, Bergstrom *et al*. (1967) compared subjects' ability to sustain cycling at 75% VO_2 max after a mixed diet (M), a high protein and fat diet (P), and a high CHO diet (C). As a result of the different diets subjects had vastly different muscle glycogen concentrations and were able to exercise for varying durations. After the M, P, & C diets subjects' average muscle glycogen concentration was 1.75, 0.63 and 3.31 g/100g wet muscle respectively. And, during the exercise to exhaustion trial subjects were able to exercise for 114, 57 and 167 minutes after the M, P, and C diets respectively. Interestingly, not only did the size of the glycogen reserves prior to

exercise dictate the amount of work possible, but those subjects who started with more glycogen preferentially utilized this fuel source. This was determined through analyzing the change in muscle glycogen concentration as revealed by muscle biopsies. As a result of the exercise bout subjects' mean glycogen scores decreased by 0.213, 0.13 & 0.261 (mmol/kg tissue/min) after M, P, and C diets. Also observed in this study was a higher resting lactate and RQ in the C diet, indicating that resting glycolysis was inflated above normal levels in this treatment.

Figure 2 is adapted from Bergstrom *et al*. (1967) and clearly shows the relationship between the initial glycogen content of the rectus femoris muscle and the work time. Notably, the highest muscle glycogen starting values were after the CHO diet, which also resulted in the longest time to fatigue in each subject. Furthermore it seems that the training status of the subject has little to do with their muscle glycogen loading ability and ability to use glycogen during the exhaustive exercise bout since in their study the best and least trained subjects performed similarly at the 75% relative intensity when given the CHO diet (2). Additionally there was a strong correlation between the amount of CHO oxidized and the decrease in muscle glycogen, which further corroborates that the muscle glycogen is an integral energy source during high intensity exercise (2).

Another classic glycogen loading study by Sherman *et al*. (1981) helped further elucidate the efficacy of different methods of glycogen supercompensation and subsequent running performance (52). They found that 3 days of a mixed diet (353g CHO/day) with a depletion protocol and then 3 days of a taper protocol with high CHO diet (542gCHO/day) increased muscle glycogen concentration. Contrary to the previously

held belief they found that the low CHO diet during the depletion period did not increase glycogen storage during the subsequent high CHO period. In agreement with other studies they found that CHO oxidation rates differed based on the starting amount of muscle glycogen. Thus, despite the fact that subjects started at different glycogen levels they ended up at approximately the same concentration (52).

Additional evidence that the pre exercise muscle glycogen status influences substrate utilization and subsequent intensity of exercise comes from Gollnick *et al*. (1981). Subjects performed 20 min of cycling at a moderate and high intensity workload separated by 1 hour of rest (28). The day prior to testing the subjects were directed through a glycogen depletion trial on one leg such that the starting muscle glycogen differed between the legs (28). Following 1 hour of rest Nicotinic acid was injected to inhibit lipolysis (28). The leg with low initial glycogen showed a greater reliance on fat (28). Additionally, the low glycogen leg extracted lactate from the blood whereas the high glycogen leg released lactate (28). Glycogen utilization was positively related to the percentage of glycogen-depleted fibers, thus supporting that the magnitude of muscle glycogen stores influences the substrate uptake (28). In further support of the influence of initial glycogen status on subsequent substrate utilization, Widdrick *et al*. had subjects complete four trials with either a high or low initial muscle glycogen concentration. Subjects received either a CHO or a non-CHO beverage during 70km cycling time trial. They observed that even during the non-CHO trial, those subjects that started with high glycogen content were able to oxidize CHO from endogenous stores (62).

A substantial body of literature supports that the initial amount of muscle glycogen influences the subject's ability to undertake high-intensity exercise. This is in large part because having a greater amount of muscle glycogen at the onset of exercise allows the body to preferentially utilize this fuel source and subsequently produce ATP at high rates. For these reasons, subjects in the current investigation will be instructed to maintain a high carbohydrate intake the two days prior to the study, while concurrently refraining from vigorous exercise. This will hopefully maximize the starting glycogen concentration and provide a more robust image of depletion to be analyzed by the MuscleSound®.

TIME COURSE OF DECLINE

The present investigation will utilize six times six-minute efforts at 90% VO_2 max with six minutes of rest in between each effort. It is hypothesized that the decline in muscle glycogen will be curvilinear, as supported by several similar protocols which revealed this pattern of depletion. In their classic study, Bergstrom *et al*. (1967) helped to further elucidate the time course of the decrease in muscle glycogen during intense cycling in working and non-working muscles (4). Their protocol involved repeated 15-minute bouts of cycling at 950kpm/min separated by 15 min of rest during which time muscle biopsies were performed. As a control the subjects performed one-legged cycling bouts, enabling them to analyze the glycogen utilization in non-working muscle groups. They found that muscle glycogen breakdown was greatest during the initial bouts of exercise and successively diminished following a semi-logarithmic decline throughout the remainder of the trials (Fig. 3). Importantly, they saw no significant change in muscle glycogen

content in non-working muscles. The authors attribute the initial steep decline to the relatively anaerobic breakdown of glycogen at the beginning of intense exercise, as evidence by a rapid increase in lactic acid. The middle part of the curve appears to be due to a balancing out of the metabolic processes, and then when the glycogen stores diminish there is an increased utilization of fat and decrease in glycogen utilization with concomitant fatigue (4).

A similar depletion pattern was observed in a study by Karlsson and Saltin (1971) using five bouts of near maximal cycling for one minute with five minutes of rest in between (43). The largest decrease in glycogen was observed after the first bout, with marginally smaller decreases each subsequent effort. Muscle lactate levels in this study reached a peak at 23mmol/kg after the first bout. We hypothesize that their will be a similar curvilinear decline in muscle glycogen after each bout in the present study.

ENDOGENOUS GLYCOGEN REPLENISHMENT

The ability to replenish muscle glycogen reserves in working muscles is essential to the ability to perform repeated high-intensity efforts over successive days. When repeated days of exercise are performed the body's stores of endogenous CHO are depleted with subsequently lower ability to maintain sustained effort (10). While exogenous sources of carbohydrate are essential to rapidly replenish these stores, the body does have an innate ability to slowly resynthesize glycogen with endogenous sources. Choi *et al*. (1994) showed that after a glycogen depleting bout of intervals, there is a higher rate of glycogen synthesis when the subject remains passive than active during recovery (8). There is controversy however, what the role of lactate or plasma glucose is

in this passive endogenous resynthesis (24). As lactate and glycolytic intermediates are produced in high quantities during high intensity exercise they are thought to play a role in muscle glycogen resynthesis post exercise in the absence of exogenous carbon sources. This appears to be contingent on the intensity of the previous bout of exercise. After medium intensity exercise when glycolytic intermediates and lactate are not produced in as high of quantities some studies have shown that lactate is primarily oxidized as a fuel source rather than resynthesized as glycogen (7, 25). During high intensity exercise, however, lactate is produced in large quantities and the current understanding is that glycogen resynthesis occurs largely as a result of the body's conversion of lactate to glycogen, despite high rates of lactate oxidation (24, 34). Hermansen *et al*. (1977) had subjects perform three one-minute duration maximal bouts of exercise where lactate levels reached 20.9 mmol/L after the third bout (34). The rate of glycogen repletion was found to be 33.6 mmol glucose units / kg/hr, in excess of four times that seen in studies by Ivy *et al*. (1988), however, this was measured after only 30 min of recovery. This led them to postulate that the enormously rapid rate of synthesis of glycogen could only be due to the intramuscular conversion of lactate to glycogen. At the most they concluded that there was 10% efflux of lactate to the circulation, and only 15% could be prescribed to oxidation within mitochondria, leaving 75% to be metabolized via other routes. The elevation of plasma insulin (a potent stimulator of glycogen synthase) post exercise could also explain the high observed synthesis rates (34).

There is also some controversy regarding the metabolic pathways responsible for post-exercise conversion of lactate into glycogen. The first proposed mechanism for this

process is the direct conversion of lactate to glycogen via a reversal of the pyruvate kinase reaction within the skeletal muscle (24). The second mechanism, which involves the Cori Cycle, entails the conversion of lactate to glucose via gluconeogenesis in the liver and kidneys (24). Once glucose is produced via this metabolic pathway it is released into circulation and taken up by skeletal muscles where it is converted to glycogen (24). The rapid endogenous synthesis of glycogen during recovery in the fasted state has been shown to be due to a dephosphorylation-mediated activation of glycogen synthase (6). Additionally, during the recovery period there is a marked reduction in the activity of glycogen phosphorylase (6). As discussed previously, there is also an increased conversion of lactate to glucose via the Cori Cycle. And, with increased contraction stimulated translocation of GLUT4 to the plasma membrane glucose is able to enter the cell for conversion to glycogen (24).

Therefore, it is possible that a limited amount of glycogen could potentially be synthesized during the rest periods in the present study, however this amount would be small in comparison to the expected rate of synthesis when carbohydrate is ingested post exercise. This small amount of endogenous resynthesis could be a result of the high intramuscular lactate concentrations reached in the present study since most subjects exercised well above their lactate threshold.

GLYCOGEN RESYNTHESIS FROM EXOGENOUS SOURCES

Athletes in competitions lasting several days are often required to repeatedly perform to their maximum ability over successive days. In competitions where athletes must qualify in a morning preliminary trial and then compete again in the evening rapid

resynthesis of muscle glycogen stores is essential for success. Several factors influence the rate at which glycogen is replenished in the liver and muscles: namely timing of ingestion, the type of CHO, the insulin response to the beverage, the quantity of CHO and whether it is ingested along with a protein supplement (37).

To maximize the rate of resynthesis of muscle glycogen CHO should be ingested as soon as possible after exercise (37). Ivy *et al*. (1988) have reported that when a CHO containing beverage is ingested immediately after exercise the rate of replenishment is 6-7 umol/g wet wt. /hr (41). However, if CHO ingestion is delayed by two hours the rate of resynthesis is only 3-4 umol/g wet wt/hr (41). If more CHO is not ingested after two hours the rate of resynthesis drops by approximately 50% due to blood insulin and glucose levels returning to pre exercise levels (37). Without exogenous CHO ingestion the rate of resynthesis is only 1-2 umol/g wet wt/hr, thus highlighting the need for immediate refueling (37). To maximize the rate of glycogen replenishment 500 to 600 g of CHO must be consumed over 24 hrs (37) and this can be administered in frequent smaller meals or larger less frequent meals (14) .

The type of CHO also plays a pivotal role in the rate of replenishment due to the body's differential handling of glucose and fructose. Fructose is metabolized mainly in the liver, and thus maximizes the rate of liver glycogen resynthesis, whereas glucose is metabolized or stored as glycogen in the muscles (5). For this reason Blom *et al*. (1987) found that the rate of muscle glycogen replenishment was nearly twice as fast when glucose or sucrose were ingested versus fructose (5). Additionally, the insulin response to glucose is much greater to that of fructose (5).

Whether or not glycogen is rapidly resynthesized is largely due to the insulin response to a CHO containing beverage (37). Insulin not only stimulates cellular uptake of glucose but also increases the activity of glycogen synthase, the rate limiting enzyme in the conversion of glucose to glycogen (36). Countering the effects of glycogen synthase, glycogen phosphorylase activity in the fasted state is depressed at the onset of recovery but increases to pre-exercise levels within 20 minutes of cessation (6).

To maximize the resynthesis of muscle glycogen, a critical amount of CHO must be consumed (36). When 0.7g/kg (body mass) and 1.4g/kg glucose were provided post exercise, the rate of synthesis was the same, but when 0.35 g/kg glucose were provided the rate was cut in half (5). Ivy *et al*. explored the effect of different concentrations of CHO beverage on the rate of resynthesis and found that there was a curvilinear pattern which plateaued at 5.5 umol/g wet wt/hr when subjects were given 1-1.5g/kg CHO per two hours (36) (Fig. 4).

Since CHO concentration of replenishment beverages above ~1.5g/kg CHO/2hrs result in varying levels of blood glucose but identical rates of resynthesis the availability of CHO is not limiting (36). In support of this hypothesis, when subjects were infused with 3g glucose/kg during 3.75 hours of recovery the rate of synthesis was the same as when subjects ingested 1.5g glucose/kg/2hr over four hours post exercise, despite varying blood glucose levels (49).

While there appears to be a maximal rate of ingestion for glucose containing beverages alone, the addition of protein to the mix has been demonstrated to increase the rate of resynthesis (36, 38, 46, 63). Arginine in combination with CHO can increase the

insulin response by five-fold, thus potentially increasing glycogen synthesis (36). However, when this hypothesis was tested by Ivy *et al*. they found that the increased rate of glycogen synthesis was due to a depressed rate of CHO oxidation rather than an increased insulin response (36). On the other hand, including protein in the CHO beverage increases glycogen synthesis by 38% when 112 g CHO and 40.7g protein are given versus just CHO over a four hour recovery period, and this was due to a synergistic insulin response (63). Along with the increased insulin response in the protein trial, there was a decrease in blood glucose levels, suggesting that the insulin mediated cellular uptake of glucose was responsible for the faster rate of synthesis (63). In the current study it is expected that there will be greater rates of glycogen synthesis than what Ivy *et al*. (2002) observed but that muscle glycogen, as measured in MS units, should not reach baseline within the allotted time of recovery.

HIGH FREQUENCY ULTRASOUND IMAGING FOR GLYCOGEN

The gold-standard for assessing muscle glycogen concentration is the Bergstrom muscle biopsy technique and subsequent chemical analysis (3). This technique is limiting in its practicality for use outside a laboratory. Ultrasound, a therapeutic and diagnostic technique, has been available to clinicians since the its inception from sonar technology after World War I (45). Ultrasound is by definition at a greater frequency than humans can hear (>20kHz), with diagnostic ultrasound in the millions of hertz and therapeutic ultrasound in the thousands of hertz. Higher frequency diagnostic ultrasound has poorer penetration than low frequency ultrasound but produces images of higher resolution (45). Ultrasonography is based on the ability of a piezoelectric crystal to generate sound waves

when an electric current is applied to it. Upon return, the sound waves are transformed into a current by the same crystal material (45). Early ultrasound machines used one crystal to generate the signal and are called A-mode ultrasound, whereas modern day machines use an array of crystals to generate the image typically associated with ultrasound (45). This more advanced setup is called B-mode, 2D or Gray-scale ultrasound. Ultrasound waves penetrate well through solids or fluids tissues, but appear as black on the screen when passing through blood or another fluid (45). The ability of ultrasound to measure glycogen, or a proxy thereof, would be immensely beneficial to researchers and those involved in monitoring athletes training status. Ultrasound is presently used in a wide array of diagnostic procedures to determine muscle pathologies. For example, ultrasound has shown promise as a diagnostic tool for glycogen storage diseases (58). In adults affected with Pompe's disease, a muscle pathology characterized by the inability to decrease muscle glycogen, there were characteristic ultrasonographic changes in all pathological muscles (59).

The ability to characterize the hydration status of muscles from ultrasonography is based on changes in the velocity of the propagating sound wave (55). Lean mass is characterized by fast propagation whereas fat mass causes waves to slow down. Ultrasound in mammalian skeletal muscle generally falls in the range of 1550-1630 m/s, with little variation occurring due to fiber type orientation (55). The velocity is mostly influenced by the molecular composition and intermolecular interactions in tissues, and only slightly effected by features such as muscle fibers and fascicle sheaths (55). This is why intramuscular water and fat composition contribute substantially to alterations in sound wave velocity. For example, a 1% change in water content can cause a 3-3.5m/s decrease in ultrasound velocity (55). Typical velocities are: 1520m/s in body temperature water, 1400-1450 m/s in tissues composed of fat, and 1700-1800 m/s in high protein

content tissues (55). There does not seem to be a large dependence of ultrasound velocity on age, however being male and having a low BMI were predictors of a higher velocity (55). Clinically, patients with edemas have lower ultrasound velocities in the lower leg musculature due to increased water content (55). Patients in this same study underwent hemodialysis with average body weight loss of 3.8kg or 5% body weight. There was a 3-4 times smaller than predicted increase in velocity after the reduction in body water, indicating that muscle tissues maintain water homeostasis even during periods of substantial body water reduction (55). Collegiate wrestlers who underwent an acute dehydration protocol were monitored with ultrasound and the -3.6% change in body weight increased ultrasound velocity by 2.18 m/s (56).

Measuring muscle glycogen concentration has historically been performed using muscle biopsies which are invasive and thus limited in their applicability. A novel algorithm, MuscleSound®, may present a fast, non-invasive and painless way of measuring muscle glycogen concentration, thus enabling more muscles to be tested quickly and repeatedly. This technology uses high-frequency ultrasound and proprietary software in a cloud-based analysis program (MuscleSound®) to purportedly assess muscle glycogen. The technology utilizes the fact that muscles are dark (hypoechoic) when full of glycogen and light (hyperechoic) when depleted. To date two studies have used high-frequency ultrasound imaging for determination of muscle glycogen content. One study from Nieman *et al*. used a 75 km cycling time trial as the exercise protocol, and measured muscle glycogen with the MuscleSound® against muscle biopsies obtained during the same trial (47). Muscle glycogen from the muscle biopsies was determined with a

coupled enzyme assay. Pre determined sites on the VL and the RF were imaged with the MuscleSound®. The three images from each site were averaged to obtain an average MuscleSound® score and scaled to 0-100. VO_2 during the trial was measured at 16 and 55km and averaged 69.6% VO_2 max. Blood lactate increased by 1.05mmol/l to 2.02mmol/l from pre to post exercise. Body weight decreased 1.45kg during the trial despite the fact that subjects were allowed to consume water ad libitum. Muscle glycogen decreased by 77.2 % in MS units, however there was significant between subject variation in the absolute quantity of muscle glycogen depletion (32 to 110mmol/kg). MuscleSound® score changes of the VL and RF correlated significantly with changes reported from muscle biopsy (R^2=0.92 and R^2=0.87 respectively). Pre exercise MuscleSound® scores correlated significantly with values obtained from muscle biopsy (R^2=0.8456 and R^2=0.8162 respectively). The subjects of the study had vastly different pre-exercise muscle glycogen values as determined by both the MuscleSound® and via muscle biopsy (Fig. 5)

These data support the possibility that the MuscleSound® technology is a valid tool for accurately assessing changes in muscle glycogen concentration, however, the authors leave open the possibility that the imaging may be picking up the water associated with glycogen rather than the glycogen per se. The decrease in subjects' body weight during their trial (1.45 kg) can largely be attributed to changes in hydration status through sweating and may present an alternative conclusion to their results – namely that the MuscleSound® technology is actually capturing changes in water content rather than

glycogen. For this reason the present study sought to control for decreases in body hydration status.

The second study, from Hill and Milan (2014) validated the MuscleSound® using a 90 minute test on a cycle ergometer at an intensity eliciting 2-3 grams of CHO oxidation per minute (35). Unlike Nieman *et al*., Hill and Milan compared MuscleSound® scores with muscle glycogen concentration obtained via muscle biopsy from the RF, and used histochemical analysis instead of coupled enzyme assays to quantify glycogen (35). The use of the RF for measuring muscle glycogen via the muscle biopsy technique is unusual as data is usually reported in the VL. Additionally, it should be highlighted that the algorithm they develop for determining the muscle glycogen concentration remains proprietary and opaque. It is still unclear what score a zero glycogen containing muscle actually is. MuscleSound® uses a proprietary feature extraction algorithm for removing the skin, fat and other connective and vascular tissue from the scans. In our own pilot work we found that obtaining multiple images was necessary as they often contained shadows caused by non-uniform skin surfaces. Often the window of analysis, which was selected by the MS algorithm, contained connective tissue rather than the intended muscle. Hill and Milan do not describe any sort of pre-analysis selection criteria in their paper. It is unclear whether these authors made any attempt to control for body hydration status, as no mention is made in the paper as to what protocol they implemented for hydration, or whether their subjects could drink ad libitum. As the trial was 90 min, it is most likely that subjects were consuming beverages, although there is also no mention of a change in weight during the trial. There is limited ability to reproduce their exact

workload as a percentage of VO_2 peak, as oxygen consumption was not monitored, and the subjects' individual VO_2 peaks were not determined.

The authors found strong correlations between pre and post exercise MuscleSound® and muscle glycogen scores for RF (R^2=0.86 and R^2=0.88). Similar to Nieman *et al*. they found that the absolute change in muscle glycogen values varied widely between subjects (109 to 6 mmol/kg) and likewise the MuscleSound® score changes were highly variable (50 to 0) (35). It is worth noting that in their study a glycogen concentration on the low end post exercise of ~30mmol/kg corresponded with a MS score of ~18. Histochemical analysis is often unable to discern changes above 80-100 mmol/kg. Ten of the 22 subjects in the study by Hill and Milan supposedly had RF glycogen concentrations >100mmol/kg, corresponding to MS values of >50. Only five subjects displayed MS values in the VL >50 in the present study, and only two were above 60. In the study by Hill and Milan, they purportedly had three subjects with MS values >80. And one subject whose muscle biopsy was above 200mmol/kg also displayed a MS value that was most likely scaled intentionally to 100. One subject displayed a MS value post exercise that was >70, a value that is higher than the starting values of every subject in the present study. The regression equation from this study suggests that for every unit increase in mmol/kg via the muscle biopsy/ histochemistry technique there is a corresponding increase of 0.46 MS units, thus highlighting the narrow window of measurement the MS is capable of discerning. When coupled with the fact that the MS measures in gradients of 5 units, a small change in MS units is of questionable quantitative validity.

METHODS

Subjects

Twelve healthy, non-smoking subjects (25.5 ± 1.25y, 74.03 ± 2.58kg, 174 ± 4.48cm) were recruited from the University of Texas at Austin campus. Exclusionary criteria included having sustained an injury in the last six months to the arms or legs or having participated in physical therapy. Subjects were asked to refrain from consumption of caffeinated or alcoholic beverages the day prior to the study. Additionally, subjects were excluded if they had weight loss (>5kg) in the three months prior to the study, having a history of hypertension, and any active kidney dysfunction. Subjects were also asked to refrain from strenuous exercise the day before the trial and to adhere to the dietary protocol described subsequently. This study was reviewed and deemed exempt by the Institutional Review Board at the University of Texas at Austin.

DESIGN

Subjects visited the lab on two occasions separated by one week. The initial visit was comprised of a submaximal and VO_{2peak} test to determine peak oxygen consumption while on the stationary cycle ergometer. Subjects took a Health Questionnaire and Physical Activity Questionnaire and were excluded at this time if deemed ineligible to participate. Subjects were required to adhere to a dietary and exercise protocol over the two days prior to the test protocol to increase total body glycogen stores, as has been previously validated (51). The day of the exercise trial subjects reported to the lab in the morning and had their nude body mass and pre exercise MS values measured after voiding. Subjects then performed high intensity intervals to elicit muscle glycogen depletion,

during which time water was given during each rest period to maintain body hydration status. This was followed by a six-hour recovery phase wherein they consumed high carbohydrate and protein beverages. MS measurements were performed after each interval and every two hours over the six-hour recovery phase to elicit the pattern of MS unit change and compare this pattern against the literature. In order to assess the qualitative validity of MS for determining muscle glycogen concentration, this exercise and recovery protocol was designed to elicit a strong signal for the algorithm to measure. High intensity intermittent exercise is an established method for the depletion of both Type I and II muscle fibers (23). Further, images were taken at the VL, RF and GS as these three muscles are known to contribute differentially to power production in cycling (21), thus enabling the comparison of the MS values against the expected pattern of depletion. And lastly, the high carbohydrate and protein beverages spaced every two hours for six hours was chosen because this recovery protocol has been validated as an effective way to maximize the rate of glycogen synthesis post exercise (40, 59). Therefore, the study was designed to elicit the greatest glycogen depletion and resynthesis signature for the MS algorithm to analyze, in order to assess its qualitative validity.

SUBMAXIMAL OXYGEN CONSUMPTION

Submaximal oxygen consumption was measured during a 20 min exercise test on an exercise cycle ergometer. The intensity of the exercise was increased every five minutes for four stages. Heart rate was simultaneously recorded and the regression lines were

plotted against each other to determine the workload needed to elicit peak oxygen consumption.

VO_2 PEAK TEST

Peak oxygen consumption while cycling was measured during a 6-12 min exercise test on a cycle ergometer. Wattage was increased after 4 min, and then every 2 min thereafter until the subject reached maximal HR, had an RER >1.1 or displayed a plateau in his VO_2 greater than 1 min. During this procedure, subjects breathed into a mouth-piece (while wearing a nose-clip) that collected and analyzed the O_2 and CO_2 content of expired air. From this we determined their oxygen consumption and identified their maximal value (VO_{2peak}). Heart rate was also measured continuously from a strap worn around their chest (Suunto, Vantaa, Finland). Peak VO_2 was determined as the highest 30-second average. Thereafter, to assist recovery approximately 10 minutes of easy exercise were performed by the subject during which time water was provided.

DIET AND ACTIVITY CONTROL

48 hours pre testing subjects were contacted via phone or email and instructed to refrain from exercise the day prior to testing. Subjects were instructed to eat a diet of 8 grams of CHO per kilogram of body weight to increase muscle glycogen. This requirement could be met by eating meals of the subject's choosing. Subjects were instructed to refrain from alcohol and caffeine for 24 hours prior to testing. They were also instructed to regularly drink water and to monitor their urine color to ensure adequate hydration the day prior to testing.

INTERVAL TESTING AND REPLETION TRIAL

Subjects reported to the laboratory the morning of testing and obtained a nude body weight on a digital scale (Ohaus, CW-11, Parsippany, NJ) after voiding. Pre exercise MuscleSound® (Denver, Colorado, USA) images were obtained at the VL, RF and GS sites after shaving and marking the sites for subsequent measurements. Six images were obtained at each site for post hoc processing. Images were obtained while the subject was positioned supine on a medical examination table for the VL and RF and prone for the GS. While cycling on a stationary ergometer the subject exercised for six intervals of six minutes at 90% VO_2 peak. Blood lactate, rate of perceived exertion (RPE), Heart Rate (HR), and expired gas measurements (VO_2) were collected during the last minute of each interval. After each interval concluded subjects were led to a medical examination table adjacent to the ergometer for MS imaging. At this time subjects were instructed to consume ~100ml of H_2O to maintain body hydration status. MS images were obtained from the VL, RF and GS sites during each rest period between intervals.

During recovery MuscleSound® images were taken at 2, 4 and 6 hrs post intervals. High CHO and PRO beverages containing 2g/kg body mass sucrose and 0.5 gram/kg protein (Vega, BC, Canada) were consumed at time 0, 2 and 4 hours post exercise. This dosage of CHO and protein was determined after previous work by Ivy *et al*. that showed beverages containing >1.5g/kg CHO and additional protein ~0.5g/kg displayed maximal rates of resynthesis when given immediately after exercise and again at 2 hours (39). In the present study we extended the recovery phase an additional 2 hours to further

characterize the ability of the MS to capture the change in muscle glycogen. Nude body weight was measured immediately after cessation of exercise and at 2, 4 and 6 hrs post exercise for determination of body hydration status.

GAS ANALYSIS:

Gas analysis was completed using oxygen and carbon dioxide analyzers (Applied Electrochemistry, Models S-3A/I and CD-3A, respectively) while the participants breathed through a one-way valve (Hans Rudolph, Kansas City, MO). Ventilation was measured via an inspiratory pneumotachometer (Hans Rudolph, Kansas City, MO).

MUSCLE GLYCOGEN CONTENT

MuscleSound® scores were determined through the use of a non-invasive high-frequency ultrasound imaging technique. MuscleSound® images were taken by a trained technician before exercise, at each rest interval of the six bouts, and 2, 4 and 6 hours post exercise. The images were at predetermined marked cites on the VL, RF and gastrocnemius/soleus (GS) muscles. The ultrasound machine uses a 12 MHz linear transducer and a standard diagnostic high resolution GE LOGIQ-e ultrasound machine (GE Healthcare, Milwaukee, WI). Images were analyzed via MuscleSound®'s proprietary cloud-based software, which quantifies a muscle glycogen score on a scale 1-100 based on the opacity of the image. A darker image is indicative of greater glycogen content versus a whiter image. The coefficient of variation for this device was 0.12 throughout testing.

BLOOD LACTATE

Lab personnel familiar with the procedures assessed Blood lactate. A drop of blood was obtained from a finger of the left hand using a Contact-Activated Lancet (BD Microtainer, Franklin Lakes, NJ, USA) and Lactate Pro Analyzer (Minami-Ku, Kyoto, Japan).

HEART RATE

Heart rate was measured continuously from a strap worn around the subject's chest (Suunto, Vantaa, Finland). Heart rate data was used as a validation of the intensity of exercise.

Statistical Analysis

A repeated measures ANOVA was used to determine significance between each time point for all measures. Differences between means were determined by a post-hoc Bonferonni test. Values are presented as mean ± SEM.

Results

VASTUS LATERALIS

There was an exponential decline in MS units measured at the VL (Figure 6). After bouts 3 through 6 VL MS values were significantly lower than versus pre exercise ($p<0.05$). During the recovery phase, two, four and six hours post exercise all showed significantly increased values versus immediately following interval six ($p<0.05$). MS values at the VL decreased 27% from pre exercise to post bout six (47.89± 3.6 to 34.86± 5.43, $p<0.001$). After two hours of recovery, there was a 26% increase relative to pre exercise values (34.89±5.43 to 47.45±4.21). MS unit restoration had surpassed the starting point by four hours with a 30.3% recovery relative to pre exercise values (34.89±5.43 to 49.41±5.43). A small decline occurred from hours four to six, with only a 27.9% recovery at six hours (34.89±5.43 to 48.24±4.7).

RECTUS FEMORIS

The RF displayed a curvilinear trend downwards with bouts 4 through 6 showing decreased MS values ($p<0.05$) (Figure 7). During the recovery phase, MS units four hours post exercise was significantly greater than the last bout of exercise ($p<0.05$). MS values at the RF decreased 21% from pre exercise to post bout six (49.40±3.18 to 39.08±3.00, $p<0.001$). After two hours of recovery, there was a 14.8% recovery versus pre exercise values (39.08±3.0 to 46.4±2.9). MS unit restoration had surpassed the starting point by four hours with a 22% increase relative to pre exercise values

(39.08±3.0 to 49.96±3.4). From hours four to six there was a decrease with only 18% recovery relative to pre exercise (39.08±3.0 to 48.11±3.4).

GASTROCNEMIUS SOLEUS

No values were significantly different from pre exercise during the intervals (Figure 8). During recovery no values were different from immediately following interval six. MS values at the GS showed a trend to decreasing 10% from pre exercise to post bout six (43.97± 2.87 to 39.70± 3.17, p=0.06). After two hours of recovery there was a 13% recovery (39.7±3.1 to 45.63±2.86). After four hours the recovery was 14% (39.7±3.1 to 46.11±2.6). After six hours the recovery was 16% (39.7±3.1 to 46.89±3.27).

BLOOD LACTATE

Blood lactate values remained the same throughout the exercise bouts ($p>0.05$) (Figure 9). Values were 10.8±0.6, 11.3±0.9, 11.1±0.7, 11.1±0.5, 11.6±0.6, & 11.2±0.7 mmol/dl for bouts one through six respectively. The average blood lactate for all trials was 11.2±0.1 mmol/dl.

VO_2

VO_2 did not significantly change throughout the trial, with mean VO_2 of 84±0.0% of maximum (3566± 101 ml/min) (Figure 10).

HEART RATE

Heart rate after interval one was significantly lower than all other time points ($p<0.05$) (Figure 11). Average heart rate was 179±1.3 bpm across all intervals.

RPE

RPE after interval one was significantly lower than bouts two through six ($p<0.05$) and displayed an upward trend (Figure 12). Average RPE across all intervals was 16.7±0.5.

BODY HYDRATION STATUS

Body weight was significantly lower (74.14 ± 2.89 vs. 73.56 ± 2.78 kg, $p<0.05$) after the exercise bouts than pre-exercise (Figure 13). After two hours body weight had increased to 74.10 ± 2.84 kg versus post exercise ($p<0.05$) and remained the same four and six hours post exercise.

Discussion

The major finding of this study is that it is inconclusive whether a new algorithm (MuscleSound®) for non-invasively measuring muscle glycogen concentration, is valid at tracking the change in muscle glycogen over repeated bouts of high intensity exercise and subsequent recovery. The present study suggests that a decline in muscle glycogen occurred in a curvilinear pattern, predominantly in the vastus lateralis and rectus femoris with no change in the gastrocnemius and soleus. Over the course of six hours post exercise, supplementation with a high carbohydrate beverage caused a significant increase in muscle glycogen concentration as measured with the ultrasound device. To our knowledge this is the first study to attempt to validate the algorithm developed by MuscleSound® using several high-intensity interval bouts and subsequent refueling. Qualitatively, in terms of detecting the correct direction of change in muscle glycogen concentration, the MS device appeared valid. Whether is appeared quantitatively valid is discussed below.

This study is also novel in the deliberate attempt to control for body hydration status. The decline from pre to post exercise in body weight, while statistically significant, is physiologically miniscule (<1.0% body weight) compared with previous validation studies which showed greater body weight changes, thus supporting the contention that any change observed in the opacity of the image is the result of glycogen concentration change and not water content. The present study is also the first to scan the ankle plantar-flexors (gastrocnemius and soleus), muscles known to display approximately half the amount of activation as the knee extensors during cycling (21).

Because the lower leg musculature displayed a no change in MS units throughout the cycling bouts this did lend credence to the qualitative validity of MuscleSound® technology to pick up glycogen in active muscles.

Over the course of six intervals of high intensity cycling MS scores in the VL and RF decreased in a curvilinear pattern. This was expected since high work rates require high rates of endogenous carbohydrate oxidation to meet energy demands (26, 30, 51, 60). It was further expected that the VL and RF would display a greater decrease in muscle glycogen than the GS as these are the main force producing muscles in cycling (21) and this was observed. Repeated high intensity bouts of exercise have been shown previously to be effective at reducing muscle glycogen content as they require both fast and slow twitch muscle fiber recruitment to achieve the forces needed (22). After repeated bouts of intermittent intense cycling, muscle glycogen in the VL has been shown to decrease from 344 to 213 mmol/kg in type I, and 402 to 203 mmol/kg dry weight in type II (corresponding to 38% and 49% declines respectively) (22). This magnitude of decline was not seen in the present study despite using a protocol that elicited a higher percentage of peak VO_2. In another study utilizing 10 min intervals at 90% VO_2 max, subjects decreased to 26, 20, 39 and 48% of post warm-up values respectively (Type I, IIA, IIAB and IIB) after only three repeats (60). We suspect because most of the subjects were not experienced cyclists or endurance athletes that their ability to push themselves through the discomfort associated with the high work rates in the present study was limited, and the work rate for several subjects had to be adjusted to allow for completion of the protocol. Despite a lower than intended average VO_2, the average RER was 1.00

+/- 0.01 with concomitant high levels of blood lactate 11.23 +/-0.1 mmol/dL, which indicates that carbohydrate oxidation was occurring at high rates. Additionally, it has been shown in the classic study by Bergstrom *et al*. (2) that the amount of glycogen the subject starts with intramuscularly can determine their time to fatigue at a given workload. Notably several subjects were not able to adequately adhere to the prescribed classic carbohydrate loading scheme described previously (52). Thus the small decline (27%) in MS units observed in the present study may be due to the untrained nature of the subjects and their low starting levels of muscle glycogen.

Because the present study was the first to attempt to control for body hydration status it may be better understood how the MS device captures the muscle glycogen concentration. Water typically accompanies muscle glycogen (48) and thus the device may simply be capturing changes in the opacity of the muscle as reflected by alterations in water content. If this is indeed the case it may address the observed increases in MS values in some subjects during intervals, contrary to what would be expected if the device only detected glycogen changes. Along those lines it has been observed that intramuscular total water content increases after commencing three bouts of three minutes of cycling at 120% VO_2 max from 313 to 359 ml/100g dry weight in the active VL muscle, with no change in the inactive triceps brachii (54). We observed a significant but physiologically small decline in body water content as a result of the exercise bouts. It is possible, as addressed above, that changes in intramuscular water content from hydrostatic pressures of muscle contraction may have affected the opacity of the image. There was a significant increase in body hydration status from post exercise to hour two

of recovery, but a non-significant increase in MS values at the GS was detected, thus supporting that changes in MS are not due to water content.

All of the previously discussed findings provide some support for the qualitative validity of the MS for capturing glycogen. During successive bouts of intense exercise mean values declined and during the first 120 min of recovery values increased. During the recovery portion of the protocol, the quantitative validity of MS becomes less clear, as MS values increased in the VL and RF as expected, but at a much faster rate than previously reported in the literature. After 120 min of recovery post a severe glycogen depleting exercise Ivy *et al*. observed 21.1% restoration with a high carbohydrate and protein beverage (39). Probably the most surprising finding in the present study was that by 120min MS values were back to baseline. If the decline in MS values observed in the present study from exercise is of the same absolute magnitude as observed by Ivy *et al*. the rate of glycogen resynthesis during the first 120 min of recovery would have to be more than three times higher than the highest value reported in the literature using direct muscle biopsies to chemically measure muscle glycogen concentration. The discrepancy between the literature and the present study could be attributed to several factors, including the possibility that the high blood lactate values achieved during the present study may have added to endogenous muscle glycogen resynthesis as has been previously observed (34). In this study Hermansen *et al*. noted rates of glycogen repletion in excess of 4 times that normally observed in CHO repletion trials, which they attributed to endogenous conversion of lactate to glycogen. Additionally, in the present study only 27% "glycogen" depletion occurred, whereas Ivy *et al*. observed a 70% decline (39). The

rapid repletion observed could be because the small amount needed to be replenished occurred in the fast stage of resynthesis, which is understood to occur initially (~45 min) during glycogen resynthesis (39). However, there is still the real possibility that the MS algorithm is indeed capturing something other than glycogen that is associated with intense exercise. The unrealistically rapid rate of "glycogen" replenishment does seem to favor this possibility.

The clear stepwise decline observed in MS units of the muscles involved in the production of power while cycling at high intensities lends credence to the efficacy of this new non-invasive MS technology. However, the rapid rate of resynthesis post exercise is much higher than previously observed and despite the intent to maintain body hydration status there was a decline after exercise, albeit of a physiologically small amount. For these reasons future studies would do well to further investigate exactly what is being measured by the ultrasound technology. One way of investigating this would be to deliberately dehydrate the subject while controlling for muscle glycogen depletion. Any changes in the MS score would then suggest that it is in fact capturing water change associated with molecules of glycogen. While not possible in the current study, using one-legged cycling and an assisted ergometer which would accurately maintain the muscle activation patterns associated with cycling would allow for the inactive leg to act as a control and further elucidate the ability of the device to detect muscle glycogen declines from exercise.

In summary, we found that repeated bouts of intense cycling followed by a six hour replenishment period displayed the expected visual qualitative pattern of muscle

glycogen's progressive decline with exercise and subsequent return during recovery following ingestion of carbohydrate and protein, as measured by a new and non-invasive ultrasound technology, MuscleSound®. However, we interpret the complete return of MS values to normal resting values with only 120 min of recovery to raise the question of whether the device is truly measuring muscle glycogen. Future studies should focus on elucidating the molecules contributing to the MS signal, with attention paid to closely controlling for body hydration status. At the moment, the MuscleSound® technology represents a step forward for the study of glycogen metabolism, as it is non-invasive and can capture numerous images of various muscle groups in rapid succession, allowing greater in-field and laboratory versatility. However, the observation that depleted MS scores can be totally replenished in only 120 min after exercise raises the question as to whether the algorithm is valid from a quantitative perspective.

FIGURES

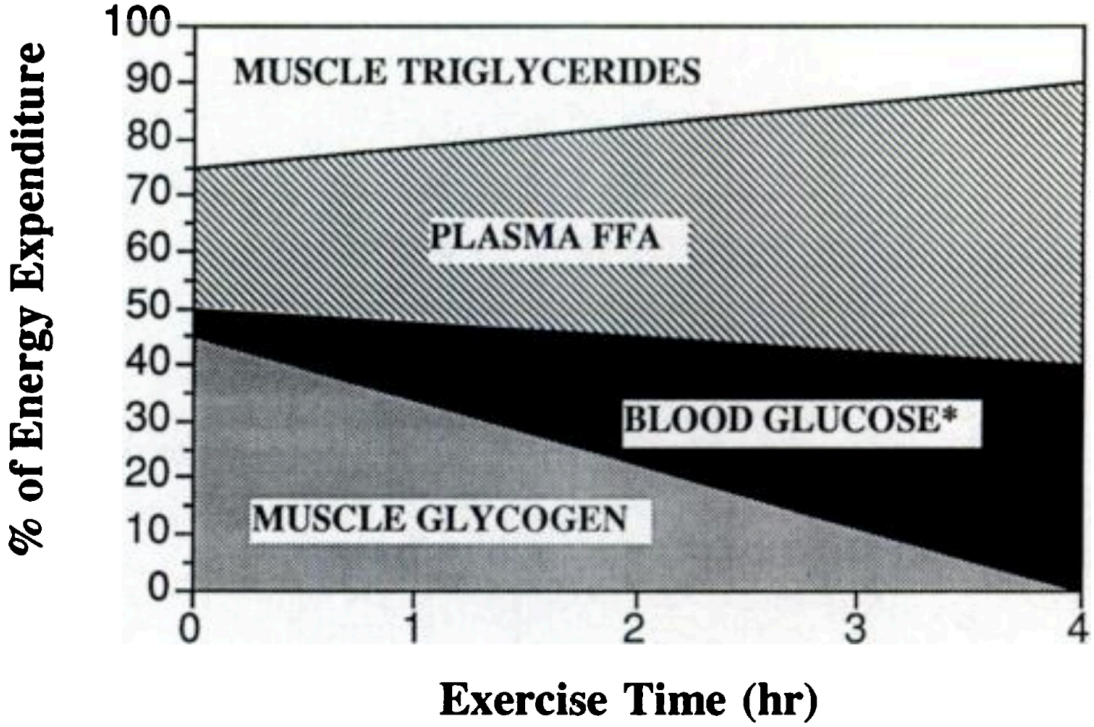

Figure 1: Substrate utilization as exercise duration increases. Adapted from Coyle (1995).

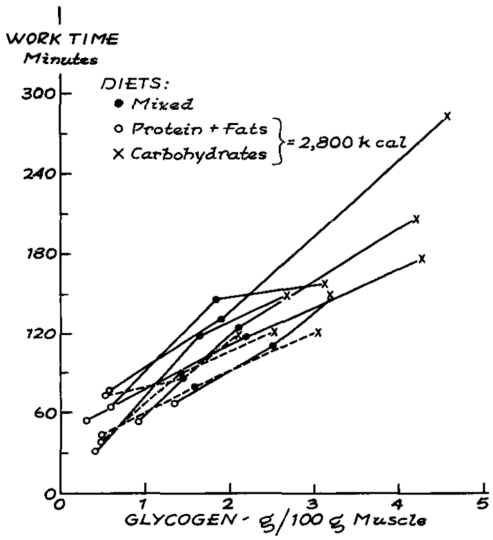

Figure 2: Relationship between initial glycogen concentration in the quadriceps and work time. Adapted from Bergstro.J, Hermanse.L, Hultman and Saltin (2)

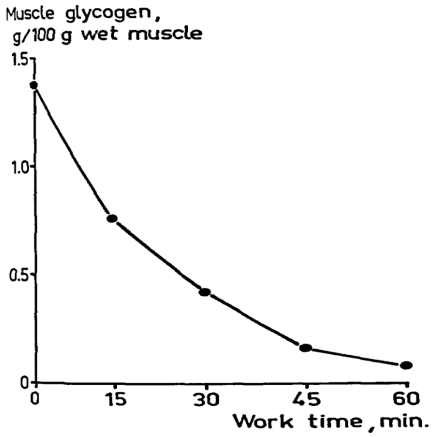

Figure 3: Pattern of muscle glycogen depletion after repeated bouts of intense cycling. Adapted from Bergstrom *et al*. (1967)

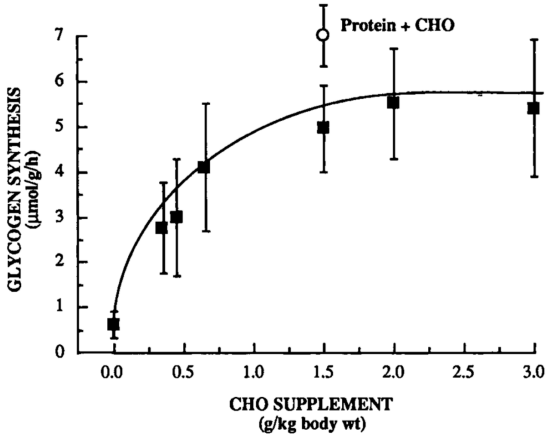

Figure 4: Effect of different amounts of CHO supplement on glycogen resynthesis rate following depleting exercise. Adapted from (36)

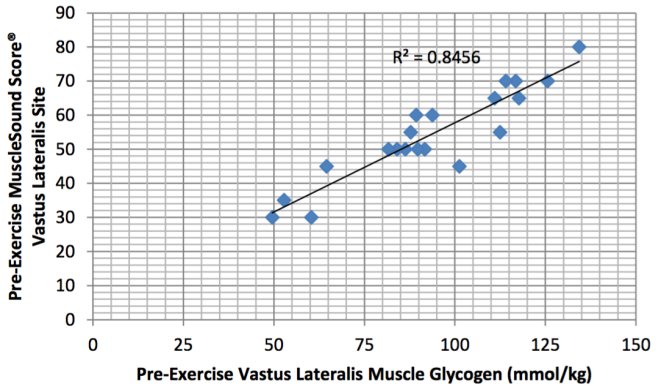

Figure 5: Regression of pre-exercise VL muscle glycogen scores as determined by muscle biopsy and MuscleSound®. From Nieman *et al*. 2015

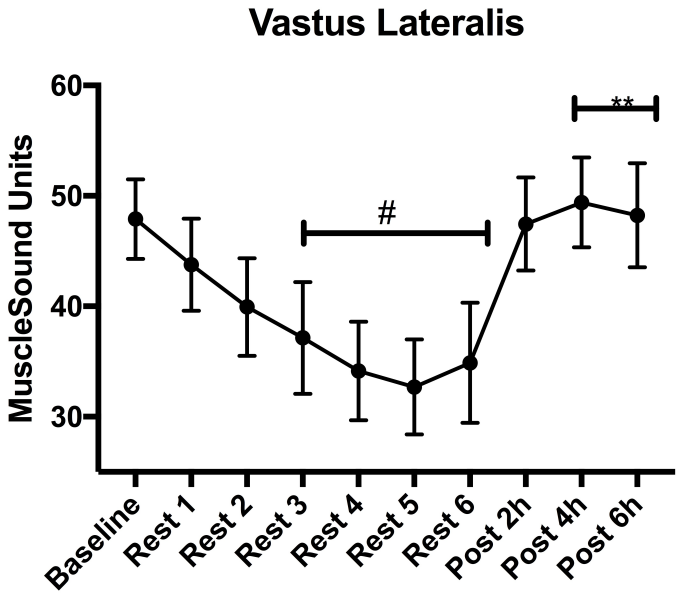

Figure 6 Vastus lateralis MS score vs. time. # 3, 4, 5 & 6 were significantly lower than pre exercise ($p<0.05$). **2, 4 & 6 hrs post exercise were significantly higher than 6th rest ($p<0.05$).

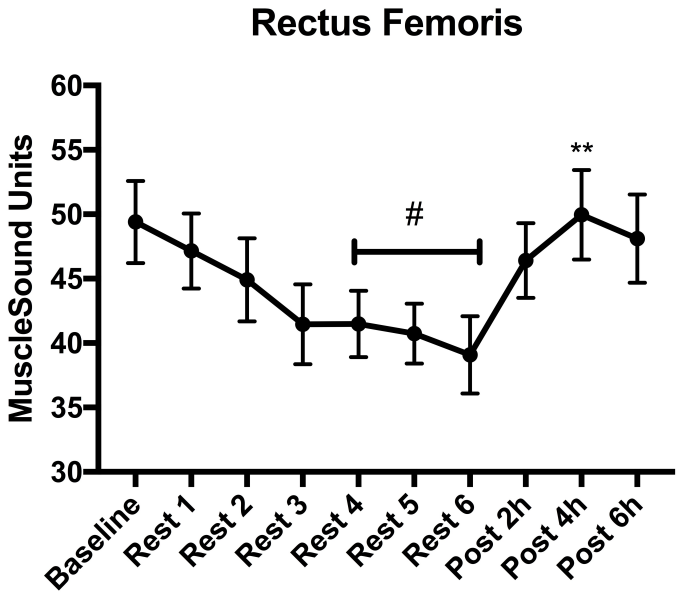

Figure 7: Rectus femoris MS score vs. time. #4, 5, & 6 were significantly lower than pre exercise ($p<0.05$). **4 hrs post exercise was significantly greater than 6th rest ($p<0.05$).

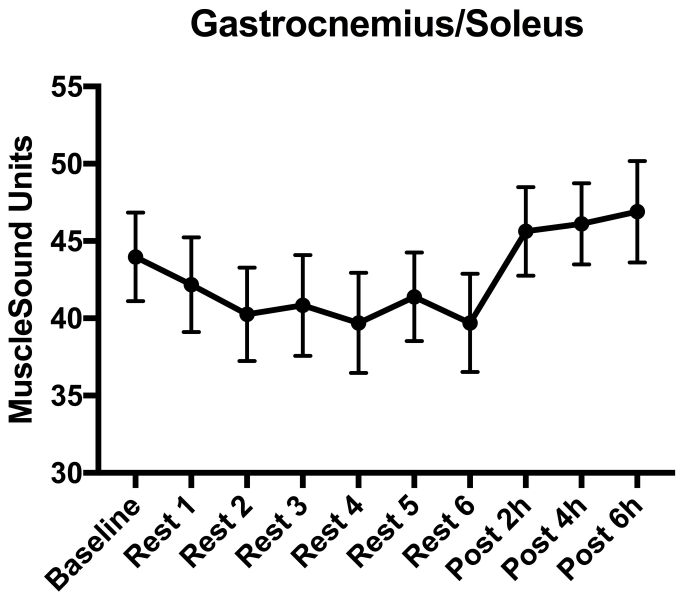

Figure 8: Gastrocnemius/Soleus MS score vs. time. No values were significantly different across time.

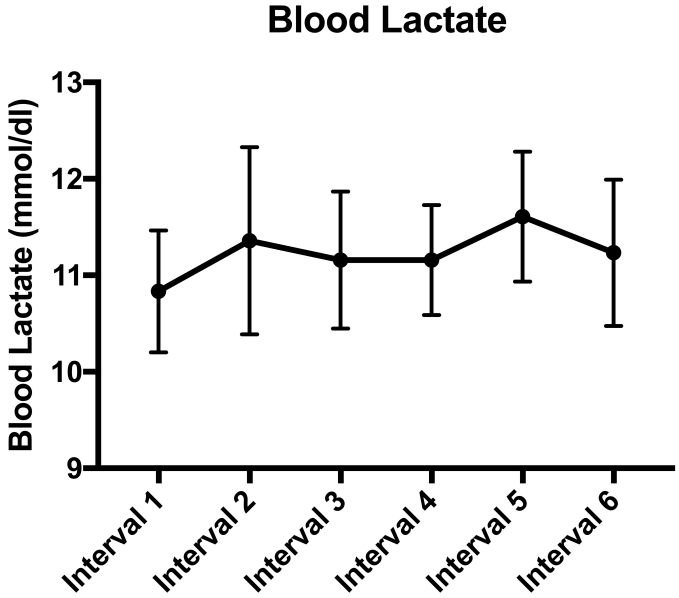

Figure 9: Blood lactate vs. time. No values were significantly different across time.

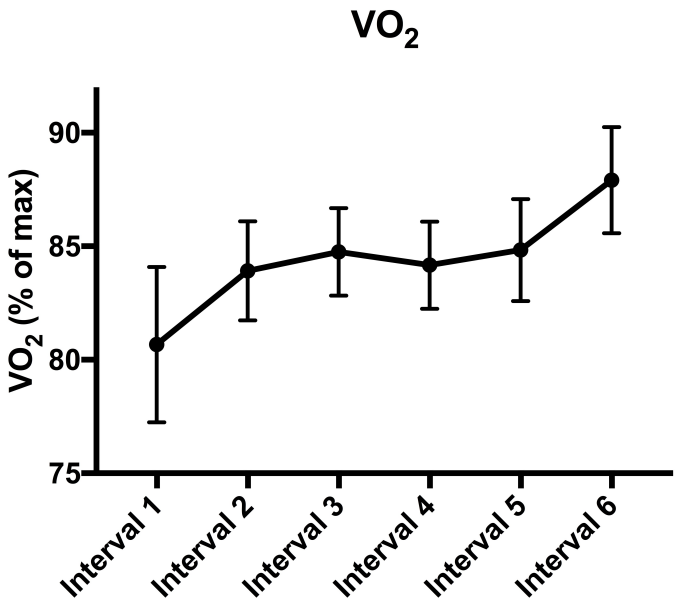

Figure 10: VO_2 vs. time. No values were significantly different across time.

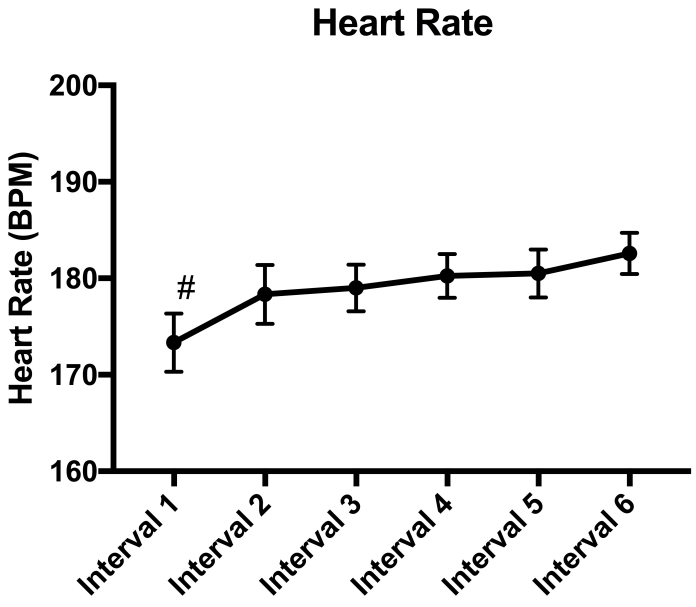

Figure 11: Heart rate vs. time. # Heart rate during interval 1 was significantly lower than all other intervals ($p<0.05$)

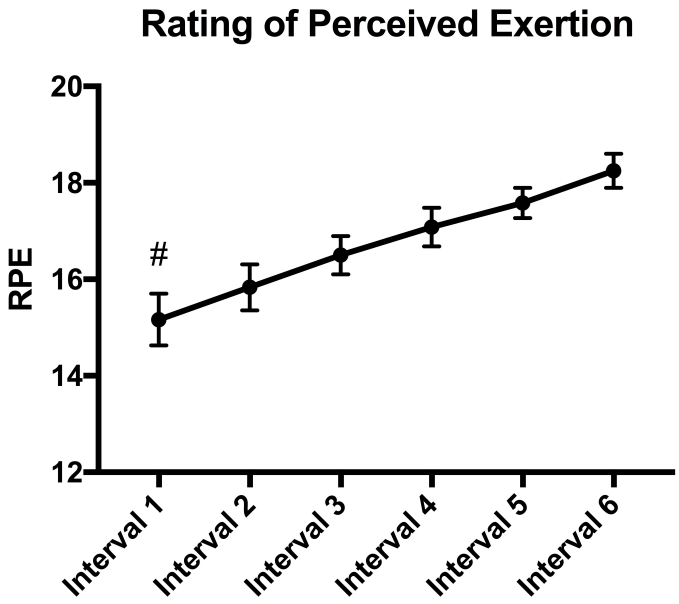

Figure 12: RPE vs. time. # RPE during interval 1 was significantly lower than all other intervals ($p<0.05$).

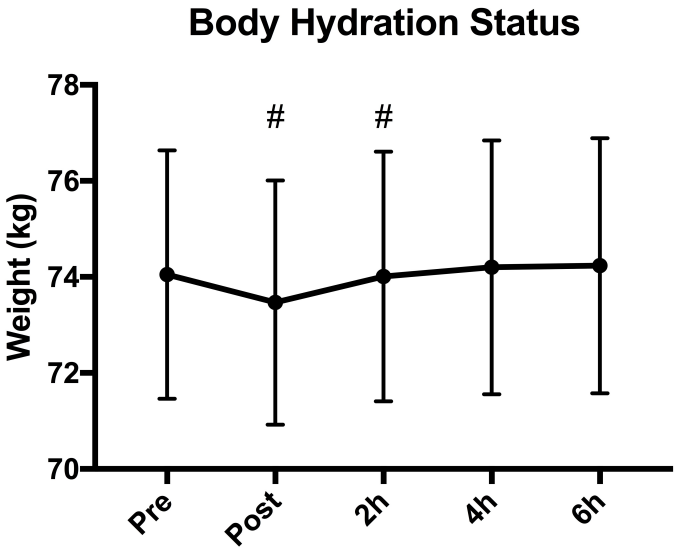

Figure 13: Body hydration status vs. time. # Body weight was significantly lower post exercise vs. pre exercise, and greater at 2 hr post exercise vs. immediately post exercise. ($p<0.05$).

Appendix

GLOSSARY

Vastus lateralis (VL)

Rectus femoris (RF)

Gastrocnemius/soleus (GS)

MuscleSound® (MS)

Rating of perceived exertion (RPE)

Heart rate (HR)

VL

Sub.	Pre	1	2	3	4	5	6	P 2h	P 4h	P 6h
1	51.25	53.75	52.50	47.50	40.00	35.00	45.00	45.00	52.50	50.00
2	50.00	46.67	46.00	43.00	38.57	43.00	42.86	56.67	48.00	54.00
3	63.33	55.00	46.67	55.00	51.67	39.00	51.67	61.25	62.50	65.00
4	34.00	30.00	27.50	12.00	13.75	7.50	2.50	29.00	35.83	34.00
5	52.50	45.00	44.00	37.00	43.00	47.14	41.67	53.00	57.50	54.00
6	38.00	33.00	32.50	30.00	30.00	30.00	35.00	43.75	50.00	26.67
7	50.00	50.00	53.57	45.00	40.00	43.00	50.00	54.00	49.17	55.00
8	58.57	50.71	45.00	38.33	41.67	47.00	45.71	60.00	57.86	58.33
9	27.00	19.38	11.82	11.67	9.17	7.22	6.11	20.00	18.33	14.50
10	44.29	38.57	32.22	40.00	33.75	32.50	35.00	38.57	45.56	48.33
11	69.17	73.00	66.25	70.00	56.88	46.00	55.63	70.71	74.44	72.85
12	36.67	30.00	21.25	16.25	11.25	15.00	7.50	37.50	41.25	46.25
Mean	**47.90**	**43.76**	**39.94**	**37.15**$^{\#}$	**34.14**$^{\#}$	**32.70**$^{\#}$	**34.89**$^{\#}$	**47.45**	**49.41****	**48.24****
STD	**12.48**	**14.44**	**15.28**	**17.53**	**15.46**	**14.93**	**18.82**	**14.58**	**14.07**	**16.28**

Vastus lateralis MS score vs. time. # 3, 4, 5 & 6 were significantly lower than pre exercise ($p<0.05$). **2, 4 & 6 hrs post exercise were significantly higher than 6th rest ($p<0.05$).

RF

Sub.	Pre	1	2	3	4	5	6	P 2h	P 4h	P 6h
1	56.25	52.50	48.33	50.00	46.67	45.00	38.75	48.75	55.00	58.00
2	50.00	50.00	42.00	47.50	45.00	45.00	47.00	51.25	48.75	46.67
3	43.33	46.67	48.33	48.33	45.00	42.00	46.00	51.67	60.00	48.00
4	38.33	43.75	34.00	32.00	32.00	28.75	31.25	37.00	35.00	38.00
5	58.75	43.75	48.75	37.50	45.00	40.00	40.00	53.75	57.50	57.50
6	40.00	40.00	38.75	26.25	33.75	35.00	36.25	42.50	41.25	26.25
7	58.33	55.00	57.00	56.00	52.50	48.00	46.00	46.00	56.25	53.75
8	58.33	56.67	50.83	50.00	50.83	49.17	49.17	62.50	63.33	61.67
9	35.00	35.00	33.33	35.00	32.50	38.00	23.75	26.00	28.33	29.38
10	60.00	50.00	53.33	51.25	49.00	47.50	55.00	52.00	62.50	53.33
11	62.50	65.00	61.67	41.25	41.88	46.67	35.83	51.67	56.67	60.83
12	32.00	27.50	22.50	22.50	23.75	23.75	20.00	33.75	35.00	44.00
Mean	**49.40**	**47.15**	**44.90**	**41.47**	**41.49**[#]	**40.74**[#]	**39.08**[#]	**46.40**	**49.97**[**]	**48.11**
STD	**11.02**	**10.11**	**11.17**	**10.76**	**8.92**	**8.07**	**10.42**	**10.04**	**12.05**	**11.84**

Rectus femoris MS score vs. time. #4, 5, & 6 were significantly lower than pre exercise ($p<0.05$). **4 hrs post exercise was significantly greater than 6th rest ($p<0.05$).

GS

Sub.	Pre	1	2	3	4	5	6	P 2h	P 4h	P 6h
1	48.75	41.25	41.67	45.00	35.00	35.00	28.75	55.00	46.67	50.00
2	40.00	45.00	43.33	46.67	40.00	46.67	43.33	46.67	42.50	46.67
3	46.67	56.67	46.67	50.00	48.75	50.00	50.00	53.33	56.00	57.50
4	56.25	45.00	45.00	48.2	48.33	51.67	52.50	51.25	57.50	53.00
5	50.00	46.67	41.25	41.67	46.67	43.75	35.00	46.25	45.00	46.00
6	25.00	27.50	30.00	27.50	28.33	30.00	30.00	31.67	28.33	28.75
7	46.25	51.25	53.33	53.33	53.75	53.75	54.00	53.75	52.50	55.00
8	58.75	58.33	55.00	57.50	55.00	51.67	53.33	56.67	55.71	63.33
9	35.71	35.00	24.29	26.25	20.63	25.00	24.17	32.50	46.67	44.00
10	37.86	30.71	26.25	33.33	25.00	28.33	27.50	38.00	37.50	28.75
11	50.00	42.50	47.50	39.17	40.00	40.83	42.86	52.50	50.00	56.00
12	32.50	26.25	28.75	28.75	35.00	40.00	35.00	30.00	35.00	33.75
Mean	**43.98**	**42.18**	**40.25**	**40.83**	**39.70**	**41.39**	**39.70**	**45.63**	**46.12**	**46.90**
STD	**9.95**	**10.63**	**10.47**	**10.80**	**11.24**	**9.92**	**11.00**	**9.93**	**9.07**	**11.36**

Gastrocnemius/Soleus MS score vs. time. No values were significantly different across time.

Blood Lactate

Sub.	1	2	3	4	5	6
1	10.4	17.7	15.7	15.8	15.8	15.3
2	12.4	7.2	11.1	12	12.8	12.1
3	14	14	13.8	10.8	12	12
4	12.4	13.4	12.6	12	12.6	12.6
5	9.1	9.2	12.3	10.9	11.6	11.6
6	10	9.5	9.4	9.1	9.4	9.5
7	9.2	8.4	9	11.4	11.2	9.8
8	13.4	13	11.2	11.2	11.2	11.2
9	9.1	13.7	10.9	9	11.2	10.5
10	6.8	6.5	5.9	8	6.3	5.2
11	13	13.6	11.3	12	14	14.8
12	10.2	10.1	10.7	11.7	11.2	10.2
Mean	**10.83**	**11.36**	**11.16**	**11.16**	**11.61**	**11.23**
STD	**2.18**	**3.36**	**2.46**	**1.98**	**2.33**	**2.62**

VO_2 (% of max)						
Sub.	1	2	3	4	5	6
1	86.1	93.2	89.1	85.2	83.7	85.1
2	92.9	84.4	88.6	82.0	83.3	87.7
3	78.4	78.2	77.3	78.0	82.1	76.3
4	69.1	68.4	71.9	69.7	66.7	69.7
5	50.9	75.4	78.3	78.3	79.5	90.1
6	76.0	78.5	79.0	81.9	82.3	85.0
7	87.1	89.8	90.7	91.0	95.1	99.4
8	88.6	86.4	84.9	85.4	79.7	92.5
9	94.4	90.9	89.0	86.8	90.7	94.1
10	79.7	87.5	86.6	89.4	89.2	89.4
11	86.2	87.6	87.2	88.8	90.9	93.5
12	78.8	88.3	94.1	94.0	93.9	93.2
Mean	80.7	84.0	84.7	84.2	84.8	88.0
STD	11.9	7.4	6.6	6.7	7.9	8.2

VO_2 vs. time. No values were significantly different across time.

Heart Rate

Sub.	1	2	3	4	5	6
1	161	186	176	177	174	175
2	175	173	176	175	175	176
3	164	167	168	167	173	175
4	172	182	185	185	185	185
5	170	168	175	175	179	180
6	191	192	192	190	192	189
7	168	171	170	176	170	181
8	179	186	185	185	178	186
9	191	196	192	194	198	197
10	164	164	169	173	173	173
11	182	183	184	186	188	191
12	163	172	176	180	181	183
Mean	**173.33#**	**178.33**	**179.00**	**180.25**	**180.50**	**182.58**
STD	**10.48**	**10.51**	**8.42**	**7.83**	**8.63**	**7.37**

Heart rate vs. time. # Heart rate during interval 1 was significantly lower than all other intervals ($p<0.05$)

RPE

Sub.	1	2	3	4	5	6
1	15	16	18	17	18	20
2	17	16	17	18	18	18
3	16	18	18	18	19	20
4	12	14	14	15	16	17
5	16	16	17	17	18	19
6	16	17	17	18	18	18
7	16	17	17	18	18	18
8	17	17	17	18	18	19
9	15	15	15	16	17	17
10	12	12	14	14	15	16
11	17	17	17	18	18	19
12	13	15	17	18	18	18
Mean	**15.17**[#]	**15.83**	**16.50**	**17.08**	**17.58**	**18.25**
STD	**1.85**	**1.64**	**1.38**	**1.38**	**1.08**	**1.22**

RPE vs. time. # RPE during interval 1 was significantly lower than all other intervals ($p<0.05$).

RER

Sub.	1	2	3	4	5	6
1	1.15	1.09	0.99	0.97	0.97	0.96
2	1.10	0.96	1.01	0.96	0.97	1.00
3	1.12	1.04	0.99	1.01	1.04	0.99
4	1.04	1.02	1.00	0.96	1.00	1.02
5	1.00	1.09	1.00	1.00	0.96	0.98
6	1.12	1.01	0.99	0.98	0.96	0.92
7	1.04	0.96	0.94	0.96	0.92	0.91
8	1.07	1.03	0.99	0.97	0.92	0.97
9	1.06	1.00	0.96	0.95	0.97	0.99
10	0.94	0.93	0.93	0.92	0.93	0.92
11	1.14	1.01	1.01	0.99	1.00	1.01
12	1.12	1.00	1.00	0.98	0.98	0.99
Mean	**1.07**	**1.01#**	**0.98#**	**0.97#**	**0.97#**	**0.97#**
STD	**0.06**	**0.05**	**0.03**	**0.02**	**0.04**	**0.04**

RER vs. Time. #RER significantly less than interval 1 ($p<0.05$).

Subject Characteristics

Sub.	Weight	Height	Age
1	77.25	144	23
2	72.9	185	21
3	80.9	182.8	28
4	89.5	148	34
5	64.15	177	31
6	56.1	162.5	21
7	78.15	187.9	23
8	67	165.1	25
9	78	184.1	30
10	67.5	177	22
11	78.1	188	22
12	78.9	186.7	26
Mean	**74.04**	**174.01**	**25.50**
STD	**8.96**	**15.54**	**4.34**

References

1. **Adamo KB, Tarnopolsky MA, and Graham TE**. Dietary carbohydrate and postexercise synthesis of proglycogen and macroglycogen in human skeletal muscle. *The American journal of physiology* 275: E229-234, 1998.
2. **Bergstro.J, Hermanse.L, Hultman E, and Saltin B**. Diet muscle glycogen and physical performance. *Acta physiologica Scandinavica* 71: 140-&, 1967.
3. **Bergstrom J**. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scandinavian journal of clinical and laboratory investigation* 35: 609-616, 1975.
4. **Bergstrom J, and Hultman E**. A study of the glycogen metabolism during exercise in man. *Scand J Clin Lab Invest* 19: 218-228, 1967.
5. **Blom PC, Hostmark AT, Vaage O, Kardel KR, and Maehlum S**. Effect of different post-exercise sugar diets on the rate of muscle glycogen synthesis. *Medicine and science in sports and exercise* 19: 491-496, 1987.
6. **Brau L, Ferreira LD, Nikolovski S, Raja G, Palmer TN, and Fournier PA**. Regulation of glycogen synthase and phosphorylase during recovery from high-intensity exercise in the rat. *The Biochemical journal* 322 (Pt 1): 303-308, 1997.
7. **Brooks GA, and Gaesser GA**. End points of lactate and glucose metabolism after exhausting exercise. *Journal of applied physiology: respiratory, environmental and exercise physiology* 49: 1057-1069, 1980.
8. **Choi D, Cole KJ, Goodpaster BH, Fink WJ, and Costill DL**. Effect of passive and active recovery on the resynthesis of muscle glycogen. *Medicine and science in sports and exercise* 26: 992-996, 1994.
9. **Coggan AR, and Coyle EF**. Reversal of fatigue during prolonged exercise by carbohydrate infusion or ingestion. *Journal of applied physiology* 63: 2388-2395, 1987.
10. **Costill DL, Bowers R, Branam G, and Sparks K**. Muscle Glycogen utilization during prolonged exercise on successive days. *Journal of applied physiology* 31: 834-&, 1971.
11. **Costill DL, Flynn MG, Kirwan JP, Houmard JA, Mitchell JB, Thomas R, and Park SH**. Effects of repeated days of intensified training on muscle glycogen and swimming performance. *Medicine and science in sports and exercise* 20: 249-254, 1988.
12. **Costill DL, Gollnick PD, Jansson ED, Saltin B, and Stein EM**. Glycogen depletion pattern in human muscle fibers during distance running. *Acta physiologica Scandinavica* 89: 374-383, 1973.
13. **Costill DL, Jansson E, Gollnick PD, and Saltin B**. Glycogen utilization in leg muscles of men during level and uphill running. *Acta physiologica Scandinavica* 91: 475-481, 1974.

14. **Costill DL, Sherman WM, Fink WJ, Maresh C, Witten M, and Miller JM**. The role of dietary carbohydrates in muscle glycogen resynthesis after strenuous running. *Am J Clin Nutr* 34: 1831-1836, 1981.
15. **Costill DL, Sparks K, Gregor R, and Turner C**. Muscle glycogen utilization during exhaustive running. *Journal of applied physiology* 31: 353-&, 1971.
16. **Coyle EF**. Substrate utilization during exercise in active people. *Am J Clin Nutr* 61: 968S-979S, 1995.
17. **Coyle EF, Coggan AR, Hemmert MK, and Ivy JL**. Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *Journal of applied physiology* 61: 165-172, 1986.
18. **Coyle EF, Coggan AR, Hemmert MK, Lowe RC, and Walters TJ**. Substrate usage during prolonged exercise following a preexercise meal. *Journal of applied physiology* 59: 429-433, 1985.
19. **Coyle EF, Hagberg JM, Hurley BF, Martin WH, Ehsani AA, and Holloszy JO**. Carbohydrate feeding during prolonged strenuous exercise can delay fatigue. *Journal of applied physiology* 55: 230-235, 1983.
20. **Coyle EF, Hamilton MT, Alonso JG, Montain SJ, and Ivy JL**. Carbohydrate metabolism during intense exercise when hyperglycemic. *Journal of applied physiology* 70: 834-840, 1991.
21. **Ericson MO, Bratt A, Nisell R, Arborelius UP, and Ekholm J**. Power Output and Work in Different Muscle Groups during Ergometer Cycling. *European journal of applied physiology and occupational physiology* 55: 229-235, 1986.
22. **Essen B**. GLYCOGEN DEPLETION OF DIFFERENT FIBER TYPES IN HUMAN SKELETAL-MUSCLE DURING INTERMITTENT AND CONTINUOUS EXERCISE. *Acta physiologica Scandinavica* 103: 446-455, 1978.
23. **Essen B, Hagenfeldt L, and Kaijser L**. Utilization of blood-borne and intramuscular substrates during continuous and intermittent exercise in man. *The Journal of physiology* 265: 489-506, 1977.
24. **Fournier PA, Fairchild TJ, Ferreira LD, and Brau L**. Post-exercise muscle glycogen repletion in the extreme: effect of food absence and active recovery. *J Sports Sci Med* 3: 139-146, 2004.
25. **Gaesser GA, and Brooks GA**. Metabolic bases of excess post-exercise oxygen consumption: a review. *Medicine and science in sports and exercise* 16: 29-43, 1984.
26. **Gaitanos GC, Williams C, Boobis LH, and Brooks S**. Human muscle metabolism during intermittent maximal exercise. *Journal of applied physiology* 75: 712-719, 1993.
27. **Galbo H, Holst JJ, and Christensen NJ**. The effect of different diets and of insulin on the hormonal response to prolonged exercise. *Acta physiologica Scandinavica* 107: 19-32, 1979.
28. **Gollnick PD**. Availability of glycogen and plasma FFA for substrate utilization in leg muscle of man during exercise. *Clinical Physiology* 1: 27-42, 1981.

29. **Gollnick PD, Armstron.Rb, Sembrowi.Wl, Shepherd RE, and Saltin B**. GLYCOGEN DEPLETION PATTERN IN HUMAN SKELETAL-MUSCLE FIBERS AFTER HEAVY EXERCISE. *Journal of applied physiology* 34: 615-618, 1973.
30. **Gollnick PD, Piehl K, and Saltin B**. Selective glycogen depletion pattern in human muscle-fibers after exercise of varying intensity and at varying pedalling rates. *J Physiol-London* 241: 45-&, 1974.
31. **Hargreaves M, Kiens B, and Richter EA**. Effect of increased plasma free fatty acid concentrations on muscle metabolism in exercising men. *Journal of applied physiology* 70: 194-201, 1991.
32. **Henneman E, Somjen G, and Carpente.Do**. Functional significance of cell size in spinal motorneurons. *Journal of neurophysiology* 28: 560-&, 1965.
33. **Hermanse.L, Hultman E, and Saltin B**. Muscle glycogen during prolonged severe exercise. *Acta physiologica Scandinavica* 71: 129-&, 1967.
34. **Hermansen L, and Vaage O**. Lactate disappearance and glycogen synthesis in human muscle after maximal exercise. *The American journal of physiology* 233: E422, 1977.
35. **Hill JC, and Millan IS**. Validation of Musculoskeletal Ultrasound to Assess and Quantify Muscle Glycogen Content. A Novel Approach. *Physician Sportsmed* 42: 45-52, 2014.
36. **Ivy JL**. Glycogen resynthesis after exercise: effect of carbohydrate intake. *International journal of sports medicine* 19 Suppl 2: S142-145, 1998.
37. **Ivy JL**. Muscle glycogen synthesis before and after exercise. *Sports Med* 11: 6-19, 1991.
38. **Ivy JL, Ding Z, Hwang H, Cialdella-Kam LC, and Morrison PJ**. Post exercise carbohydrate-protein supplementation: phosphorylation of muscle proteins involved in glycogen synthesis and protein translation. *Amino Acids* 35: 89-97, 2008.
39. **Ivy JL, Goforth HW, Damon BM, McCauley TR, Parsons EC, and Price TB**. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *Journal of applied physiology* 93: 1337-1344, 2002.
40. **Ivy JL, Goforth HW, Jr., Damon BM, McCauley TR, Parsons EC, and Price TB**. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *Journal of applied physiology* 93: 1337-1344, 2002.
41. **Ivy JL, Katz AL, Cutler CL, Sherman WM, and Coyle EF**. Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. *Journal of applied physiology* 64: 1480-1485, 1988.
42. **Karlsson J, and Saltin B**. Diet, muscle glycogen, and endurance performance. *J Appl Physiol* 31: 203-206, 1971.
43. **Karlsson JaBS**. Oxygen deficit and muscle metabolites in intermittent exercise. *Acta physiologica Scandinavica* 82: 115-122, 1971.
44. **Keizer HA, Kuipers H, Vankranenburg G, and Geurten P**. Influence of liquid and solid meals on muscle glycogen resynthesis, plasma fuel hormone

response, and maximal physical working capacity. *International journal of sports medicine* 8: 99-104, 1987.
45. **Moore CL, and Copel JA**. Point-of-care ultrasonography. *The New England journal of medicine* 364: 749-757, 2011.
46. **Moore DR**. Nutrition to Support Recovery from Endurance Exercise: Optimal Carbohydrate and Protein Replacement. *Current Sports Medicine Reports* 14: 294-300, 2015.
47. **Nieman DC, Shanely RA, Zwetsloot KA, Meaney MP, and Farris GE**. Ultrasonic assessment of exercise-induced change in skeletal muscle glycogen content. *BMC Sports Sci Med Rehabil* 7: 9, 2015.
48. **Olsson KaBS**. Variation in total body water with muscle glycogen changes in man. *Acta physiologica Scandinavica* 80: 11-18, 1970.
49. **Reed MJ, Brozinick JT, Jr., Lee MC, and Ivy JL**. Muscle glycogen storage postexercise: effect of mode of carbohydrate administration. *Journal of applied physiology* 66: 720-726, 1989.
50. **Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, and Wolfe RR**. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *The American journal of physiology* 265: E380-391, 1993.
51. **Romijn JA, Coyle EF, Sidossis LS, Rosenblatt J, and Wolfe RR**. Substrate metabolism during different exercise intensities in endurance-trained women. *Journal of applied physiology* 88: 1707-1714, 2000.
52. **Sherman WM, Costill DL, Fink WJ, and Miller JM**. Effect of exercise-diet manipulation on muscle glycogen and its subsequent utilization during performance. *International journal of sports medicine* 2: 114-118, 1981.
53. **Sherman WM, Plyley MJ, Sharp RL, Vanhandel PJ, Fink WJ, McAllister RM, and Costill DL**. Muscle glycogen and its apparent association with water. *International journal of sports medicine* 2: 272-272, 1981.
54. **Sjogaard G, and Saltin B**. Extra- and intracellular water spaces in muscles of man at rest and with dynamic exercise. *The American journal of physiology* 243: R271-280, 1982.
55. **Topchyan A, Tatarinov A, Sarvazyan N, and Sarvazyan A**. Ultrasound velocity in human muscle in vivo: Perspective for edema studies. *Ultrasonics* 44: 259-264, 2006.
56. **Utter AC, McAnulty SR, Sarvazyan A, Query MC, and Landram MJ**. Evaluation of Ultrasound Velocity to Assess the Hydration Status of Wrestlers. *Journal of Strength and Conditioning Research* 24: 1451-1457, 2010.
57. **van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, and Wagenmakers AJ**. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *The Journal of physiology* 536: 295-304, 2001.

58. **Verbeek RJ, Sentner CP, Smit GP, Maurits NM, Derks TG, van der Hoeven JH, and Sival DA**. Muscle Ultrasound in Patients with Glycogen Storage Disease Types I and III. *Ultrasound Med Biol* 42: 133-142, 2016.
59. **Vill K, Schessl J, Teusch V, Schroeder S, Blaschek A, Schoser B, and Muller-Felber W**. Muscle ultrasound in classic infantile and adult Pompe disease: a useful screening tool in adults but not in infants. *Neuromuscul Disord* 25: 120-126, 2015.
60. **Vollestad NK, and Blom PCS**. EFFECT OF VARYING EXERCISE INTENSITY ON GLYCOGEN DEPLETION IN HUMAN-MUSCLE FIBERS. *Acta physiologica Scandinavica* 125: 395-405, 1985.
61. **Widrick JJ, Costill DL, Fink WJ, Hickey MS, McConell GK, and Tanaka H**. Carbohydrate feedings and exercise performance - effect of initial muscle glycogen concentration. *Journal of applied physiology* 74: 2998-3005, 1993.
62. **Widrick JJ, Costill DL, Fink WJ, Hickey MS, McConell GK, and Tanaka H**. Carbohydrate feedings and exercise performance: effect of initial muscle glycogen concentration. *Journal of applied physiology* 74: 2998-3005, 1993.
63. **Zawadzki KM, Yaspelkis BB, 3rd, and Ivy JL**. Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *Journal of applied physiology* 72: 1854-1859, 1992.